

# Faecal mucinase activity assessed in inflammatory bowel disease using $^{14}\text{C}$ threonine labelled mucin substrate

A D Dwarakanath, B J Campbell, H H Tsai, D Sunderland, C A Hart, J M Rhodes

## Abstract

Previous studies have shown the presence in faeces of sulphatases, sialidases, glycosidases, and proteases relevant to mucus degradation, but the relative role of these enzymes in the degradation of colonic mucus has been unclear. A total mucinase assay using  $^{14}\text{C}$  threonine biologically labelled human colonic mucin as substrate was therefore developed in this study. Faecal mucinase activity of a pooled normal faecal filtrate was capable of removing 80% of the  $^{14}\text{C}$  threonine label from mucin within eight hours incubation, but 20% remained intact despite prolonged incubation. The pH profile of mucinase activity is broad (pH 4.5-9.5) suggesting contribution from multiple enzymes. Mucinase activity was reduced by preincubation with 100  $\mu\text{g}/\text{ml}$  chymostatin (82.8%), 0.5 mg/ml EDTA (91.6%), and 4 g/l bismuth subsalicylate (72.0%). All 55 faecal samples studied contained detectable mucinase activity, measured as dpm release/ $\mu\text{g}$  protein/hour, which was greater in samples from patients with ulcerative colitis ( $n=17$ , median 52.7, interquartile range 32.9-66.9), than controls ( $n=26$ , 34.4, 26.8-40.4,  $p<0.02$ ) or patients with Crohn's disease ( $n=12$ , 35.5, 17.5-55.7,  $p<0.05$ ). There was, however, no significant difference in faecal mucinase activity between inactive and active ulcerative colitis. These results suggest that faecal mucinase activity is one factor contributing to the thin mucus layer in ulcerative colitis and represents a potential target for drug treatment.

(Gut 1995; 37: 58-62)

Keywords: faecal mucinase activity, inflammatory bowel disease, mucin substrate.

Colonic mucus is an adherent, water insoluble gel that has several functions, including protection of the epithelium from mechanical trauma, toxins, allergens, and from microbial invasion.<sup>1</sup> It is also a potential energy source for bacteria,<sup>2</sup> which secrete a range of mucin degrading sulphatases,<sup>3</sup> sialidases,<sup>4</sup> glycosidases,<sup>5</sup> and proteases.<sup>4,6,7</sup> The relative role of these enzymes in the degradation of human colonic mucin has been unclear, but it has been speculated that a change in the relation between secreted mucus and its degradation by bacterial enzymes could be an important pathogenic mechanism in inflammatory bowel

disease.<sup>8</sup> A novel total mucinase assay has therefore been developed using as substrate human colonic mucin with  $^{14}\text{C}$  threonine incorporated into the mucin core protein. This has then been used to assess the total mucin degrading activity of faecal samples from ulcerative colitis, Crohn's disease, and normal controls.

## Methods

### Preparation of $^{14}\text{C}$ labelled mucin substrate

Rectal biopsy specimens were obtained with informed consent from five patients undergoing routine colonoscopy who were found to have macroscopically and histologically normal rectal mucosa (diverticular disease  $n=2$  and irritable bowel syndrome  $n=3$ ). Ethical permission was granted by the Royal Liverpool University Hospital Trust. The specimens were then cultured, using the method of MacDermott *et al.*,<sup>9</sup> in the presence of 10  $\mu\text{Ci}/\text{ml}$   $^{14}\text{C}$  threonine (ICN, High Wycombe, UK) to achieve biological radiolabelling of colonic mucin. Culture was for 24 hours at 37°C in 95%  $\text{O}_2/5\%$   $\text{CO}_2$  using 1 ml RPMI and 10% fetal calf serum (v/v) (Gibco, Uxbridge, UK) for each specimen, to which was added 100  $\mu\text{g}$  gentamicin/ml and 60  $\mu\text{g}$  nystatin/ml. The specimens were then sonicated with 4 $\times$ 15 second bursts on a MSE ultrasonicator (MSE Instruments, Crawley, UK). The ultrasonicate was then centrifuged for 30 minutes at 20 000  $g$  and the supernatant desalted using a PD10-Sephadex G25 M (5 $\times$ 1.5 ml) gel filtration column (Pharmacia, Uppsala, Sweden) and freeze dried. Mucin was purified using one step high performance gel filtration (Superose 6, Pharmacia, Uppsala, Sweden).<sup>10</sup> Parallel experiments showed that this yields mucin that is free from non-mucin glycoproteins as defined by buoyant density, electrophoretic mobility, and absence of detectable concanavalin A binding (mannose containing) material. All experiments used the same batch of  $^{14}\text{C}$  labelled mucin. The stock substrate had an activity of 55 dpm/ $\mu\text{g}$  protein.

### Faecal samples

Freshly voided faeces from 17 patients with ulcerative colitis, 12 patients with Crohn's disease, six with ileocolonic disease, and six with ileal disease, and 26 normal controls were stored at -80°C until assay. All of the ulcerative colitis patients were receiving a 5-amino-salicylic acid containing preparation, nine

## Departments of Medicine

A D Dwarakanath  
B J Campbell  
H H Tsai  
J M Rhodes

and Medical Microbiology  
D Sunderland  
C A Hart

University of Liverpool, Liverpool

Correspondence to:  
Professor J M Rhodes,  
Department of Medicine,  
University of Liverpool,  
PO Box 147, Liverpool  
L69 3BX.

Accepted for publication  
10 November 1994

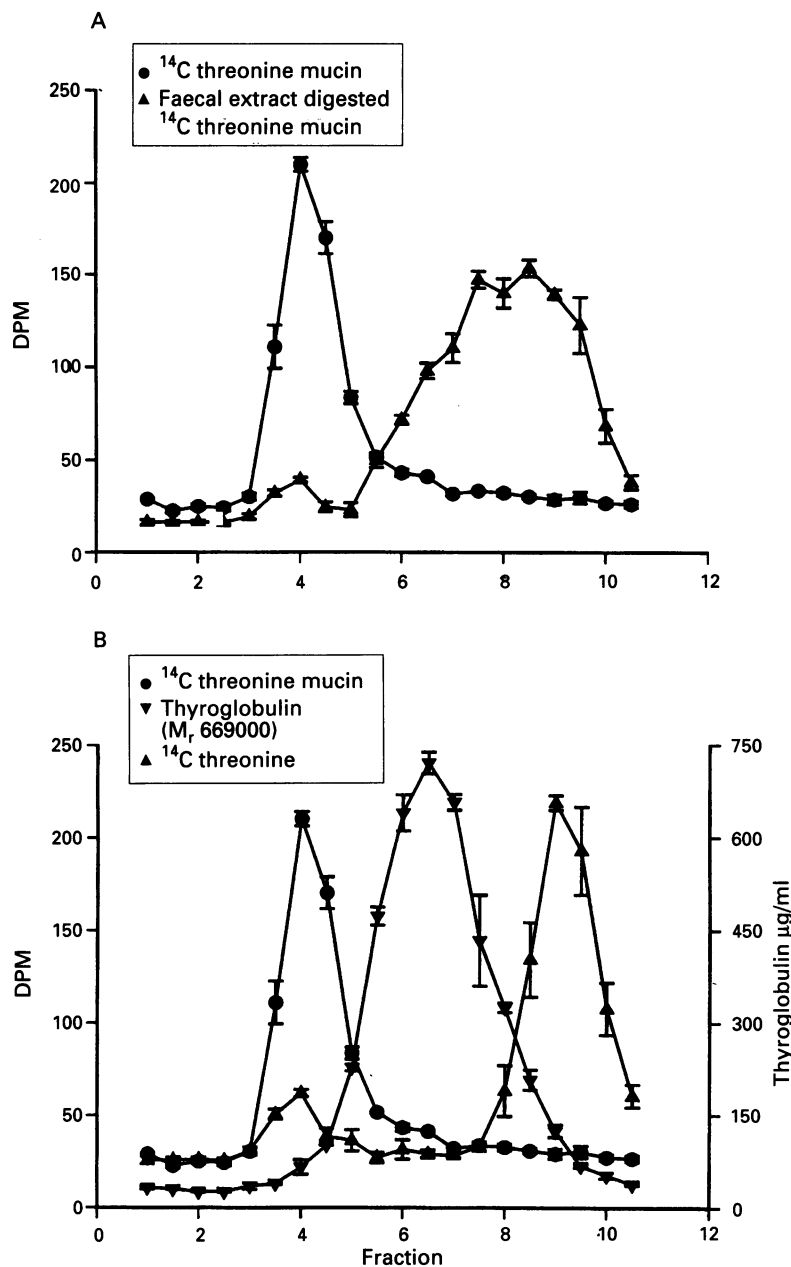


Figure 1: (A) Calibration of a CL4B gel filtration mini-column showing good separation of intact  $^{14}\text{C}$  mucin from smaller mucin fragments after incubation with a pooled faecal extract; (B) calibration of a CL4B gel filtration mini-column showing good separation of intact  $^{14}\text{C}$  mucin from smaller compounds.

taking sulphasalazine. Diagnoses of Crohn's disease or ulcerative colitis were based on conventional pathological and radiological criteria. In patients with Crohn's disease the disease activity was assessed by the Harvey and Bradshaw activity index<sup>11</sup> and ulcerative colitis by the Truelove and Witts scale.<sup>12</sup>

Faecal extracts were then prepared by homogenisation of roughly 1–2 g wet weight in 20 ml 0.05 M pH 6.5 TRIS/maleate buffer, followed by centrifugation at 20 000 *g* for 30 minutes, and filtration of the supernatant through a 0.2  $\mu\text{m}$  pore diameter filter (Millipore, Molsheim, France). Protein assays were performed using the Lowry method<sup>13</sup> on the bacteria free filtrates.

#### Total mucinase assay

Purified  $^{14}\text{C}$  mucin was diluted to 2500 dpm/ml in 0.05 M pH 6.5 TRIS/maleate

buffer. Faecal extracts (200  $\mu\text{l}$ ) were incubated with 200  $\mu\text{l}$  of the substrate for eight hours at 37°C. Each sample was tested in triplicate. The reactants were then subjected to gel filtration using gravity fed PD10 mini-columns (5  $\times$  1.5 cm) packed with Sepharose CL4B (Pharmacia, Uppsala, Sweden) and eluted with 11 ml of 0.05 M pH 6.5 TRIS/maleate buffer. The intact mucus glycoprotein eluted first in the void volume with clear separation from smaller mucin fragments (Fig 1A). Fractions of 0.5 ml were collected, and each mixed with 5 ml of scintillant (Optiphase-Safe; Pharmacia, Uppsala, Sweden) and  $\beta$  counted.

The time course, pH profile, and dose response curve for faecal mucinase activity was studied using a faecal sample pooled from six normal faecal homogenates.

#### Calibration of CL4B gel filtration columns

The gel filtration columns containing Sepharose CL4B were calibrated using purified  $^{14}\text{C}$  threonine labelled mucin, thyroglobulin ( $M_r$  669 000), and  $^{14}\text{C}$  threonine (Fig 1B).

#### Inhibitor assays

The effect of protease inhibitors on mucinase activity was assessed by preincubation of the inhibitors with the faecal extract for one hour at 37°C, followed by  $^{14}\text{C}$  mucin assay. The specific protease inhibitors used were anti-papain dihydrochloride (50  $\mu\text{g/ml}$ ; inhibits papain, trypsin, cathepsin A and B), 4-aminodiphenylmethanesulfonylfluoride (APMSF, 40  $\mu\text{g/ml}$ ; inhibits serine proteases), aprotinin (2  $\mu\text{g/ml}$ ; inhibits serine proteases), bestatin (40  $\mu\text{g/ml}$ ; inhibits amino peptidase), chymostatin (100  $\mu\text{g/ml}$ ; inhibits,  $\alpha$ ,  $\beta$ ,  $\gamma$ ,  $\delta$  chymotrypsin), E-64 (10  $\mu\text{g/ml}$ ; inhibits cysteine proteases), EDTA (0.5 mg/ml; inhibits metalloproteases), leupeptin (0.5  $\mu\text{g/ml}$ ; inhibits serine and cysteine proteases), pepstatin (0.7  $\mu\text{g/ml}$ ; inhibits aspartate proteases), and phosphoramidon (330  $\mu\text{g/ml}$ ; inhibits metalloendopeptidases). All of these inhibitors were obtained from Boehringer Mannheim, Lewes, UK. The effects of bismuth subsalicylate (4 g/l) on mucinase activity was also assessed in view of the therapeutic effect of this bismuth salt in ulcerative colitis<sup>14</sup> and its general property, as for other heavy metal salts, of enzyme inhibition.

The intra-assay and interassay coefficient of variations for mucinase assay were 5.0% and 12.0%. The recovery of  $^{14}\text{C}$  threonine within the assay was 94% (SD 3.5%). Statistical comparison between disease groups was performed using the Mann-Whitney U test and comparison between control and inhibitors using Kruskal-Wallis analysis of variance (all *p* values two tailed).

#### Results

All faecal samples had detectable mucinase activity assessed using  $^{14}\text{C}$  threonine mucin as substrate (Fig 2). Faecal mucinase activity of a pooled normal filtrate was capable of removing

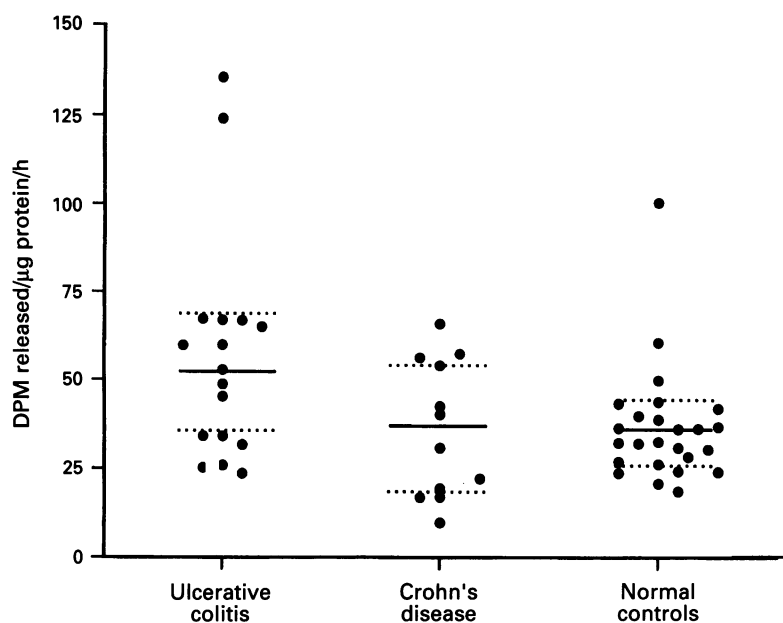


Figure 2: Faecal mucinase activity in samples from patients with inflammatory bowel disease and controls, showing the median (—) and interquartile range (...).

80% of the  $^{14}\text{C}$  label from mucin within approximately eight hours, but 20% remained intact despite prolonged incubation (Fig 3). A small proportion of free  $^{14}\text{C}$  threonine was excluded on sepharose 4B (Fig 1B), which presumably results from non-specific binding and could explain this apparent resistance to total degradation. Figure 4 shows the 'dose response' for faecal mucinase activity. The pH profile of mucinase was broad with similar activity between pH 4.5–9.5 (Fig 5). Heat inactivation (80°C for 15 minutes) of the faecal extract completely abolished mucinase activity. Mucinase activity was significantly diminished by preincubation with chymostatin and EDTA (81.8% (interquartile range 46–100) and 91.6% (74.5–100),  $p < 0.05$ ), compared with controls. Bismuth subsalicylate also produced significant inhibition of mucinase activity (72.0% (33.0–100)),  $p < 0.05$  (Fig 6).

Faecal mucinase activity, expressed as dpm released per  $\mu\text{g}$  protein per hour, was significantly higher in the ulcerative colitis group (median 52.7, interquartile range 32.9–66.9) than in the control group (34.4, 26.8–40.4,

$p < 0.02$ ) or in Crohn's disease (median 35.5, 17.5–55.7,  $p < 0.05$ ) (Fig 2). There was no difference in faecal mucinase activity between the inactive and active ulcerative colitis groups (median 48.6, 25.9–65.0,  $n = 7$  and 56.3, 23.6–135.4,  $n = 10$ ,  $p = 0.64$ ) (Fig 7).

## Discussion

In this study the use of  $^{14}\text{C}$  threonine human colonic mucin as substrate for faecal enzymes combined with Sepharose 4B chromatography of the reaction products provides a reasonable approximation to the situation in vivo. It showed the presence of mucin degrading activity in all the faecal samples studied. The very broad pH range of activity suggests that multiple enzymes participate in this process. This is similar to the broad pH range reported for faecal protease activity assessed by release of new *N*-terminals from succinyl-albumin.<sup>6</sup> The assay differs importantly, however, in its response to addition of inhibitors. The *N*-terminal release assay, which was performed at slightly higher pH (7.5) is unaffected by EDTA,<sup>6</sup> whereas the  $^{14}\text{C}$  threonine mucin assay reported here is  $>80\%$  inhibitable. This may perhaps reflect the fact that certain mucin subspecies, for example MUC2 (which is probably the commonest colonic mucin species),<sup>15</sup> have such a long ( $M_w > 1 \times 10^6$  kDa) and extensively glycosylated protein core that it will probably still be excluded from Sepharose 4B after protease attack alone and would require further or alternative degradation of its oligosaccharide chains by the appropriate sulphatase, *O*-acetyl esterase, sialidase, and glycosidase enzymes before inclusion on Sepharose 4B. It therefore seems probable that the  $^{14}\text{C}$  threonine mucinase assay reflects a combination of protease, sulphatase, and glycosidase activity and arguably gives a more complete reflection of in vivo faecal mucus degrading activity. It reflects important enzymatic disruption of the mucin, so it could be argued that biologically important but more subtle damage, for example, changes in polymeric structure and mucus rheology, may be undetected by this technique.

The increased mucolytic activity demonstrable in faecal samples from patients with

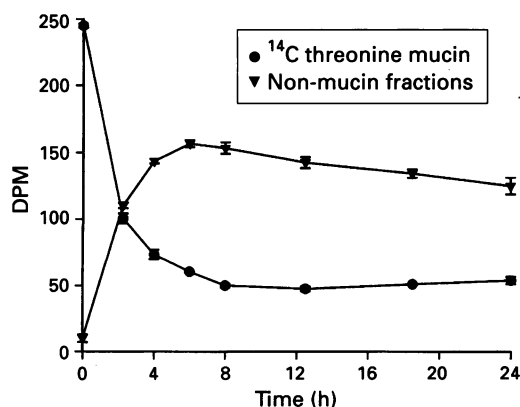


Figure 3: The time course of faecal mucinase activity showing maximal degradation of  $^{14}\text{C}$  mucin by eight hours, with 20% of the mucin threonine remaining in high molecular weight glycoprotein.

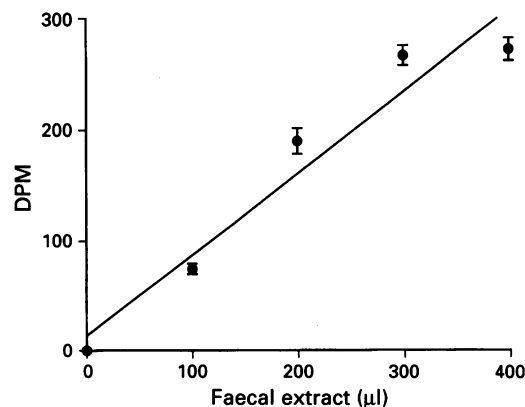


Figure 4: Mucinase activity of serially diluted pooled normal faecal extract using  $^{14}\text{C}$  threonine mucin showing  $^{14}\text{C}$  threonine release (dpm) after eight hours incubation.

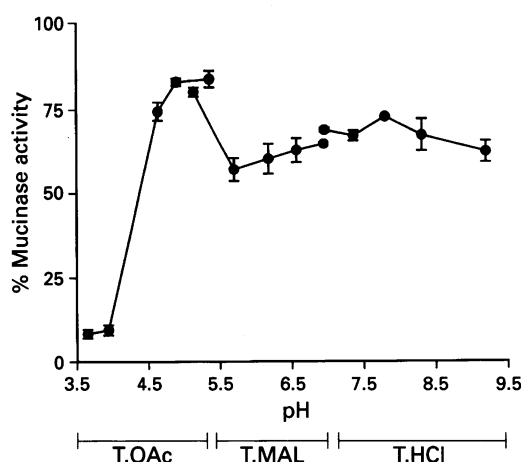


Figure 5: The pH profile of faecal mucinase activity is broad, probably reflecting the presence of several enzymes. (T.OAc=0.05 M TRIS/acetate buffer, T.Mal=0.05 M TRIS/maleate buffer, T.HCl=0.1 M TRIS/hydrochloride buffer).

ulcerative colitis complements the previously reported findings of increased faecal protease activity using azocasein as substrate<sup>4</sup> and measured by release of new *N*-terminals from succinyl-albumin.<sup>7</sup> The lack of correlation between mucinase activity and disease activity is slightly surprising, but has not been considered in the previous published studies. It can be concluded from this that relapse of colitis does not usually result from an increase in faecal mucolytic activity.

The maintained increase in faecal mucolytic activity shown in ulcerative colitis even in remission probably contributes to the abnormally thin mucus gel layer recently described.<sup>16</sup> In this respect it is interesting that the faecal mucolytic activity in Crohn's disease is

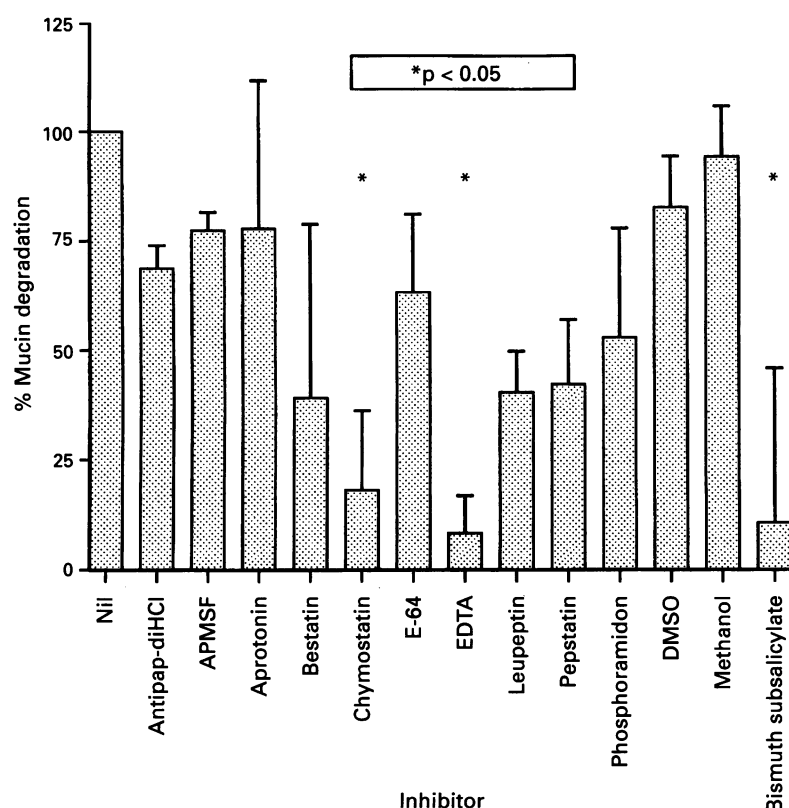


Figure 6: Effect of protease inhibitors and bismuth salts on faecal mucinase activity.

comparatively low (not significantly low compared with controls but significantly lower than in ulcerative colitis), which is in keeping with the comparatively thick mucus layer seen in Crohn's disease.<sup>16</sup>

The source of the mucolytic activity measured in the <sup>14</sup>C threonine mucin assay is unknown and is probably multifactorial. Pancreatic proteases tend to have well defined alkaline pH optima,<sup>6</sup> so probably contribute comparatively little to the mucinase activity at faecal pH, although the inhibiting effect of the chymotrypsin inhibitor, chymostatin, is perhaps surprising. The high degree of inhibition seen with both EDTA (92% inhibition) and chymostatin (82% inhibition) may be explained by the calcium dependency of chymotrypsin (or other chymostatin inhibitable proteases), which has previously been shown with certain substrates.<sup>17</sup> Leucocytes do not produce sialidases<sup>4</sup> or sulphatases<sup>3</sup> that are capable of acting on mucin as a substrate, but it is not known whether leucocyte proteases may do so. It seems probable that much of the mucus degrading activity is bacterial in origin. Faecal bacteria have been shown to secrete the spectrum of glycosidases<sup>5</sup> and sulphatases<sup>3</sup> relevant for mucus degradation and are the source of much of the protease activity in faeces.<sup>18</sup>

The persistent increase in faecal mucinase activity seen in inactive ulcerative colitis might (a) simply reflect increased substrate availability caused by increased mucus shedding (although there is no direct evidence for this); (b) reflect a reduction in faecal protease inhibitors, such as the mucin associated protease inhibitor<sup>19</sup> and  $\alpha$  1 antitrypsin,<sup>20</sup> both of which are secreted with mucus; alternatively (c) it might imply a subtle change in faecal flora, which has not so far been obtained by conventional microbiological techniques, excepting specific examples such as the increase in adhesive *Escherichia coli*.<sup>21</sup> If this is the case it could explain the anecdotal reports of apparently dramatic therapeutic response to the rather bizarre treatment of faecal transplantation.<sup>22</sup>

Whether or not the faecal mucinase activity has a direct role in pathogenesis, it provides a very plausible target for effective treatment.

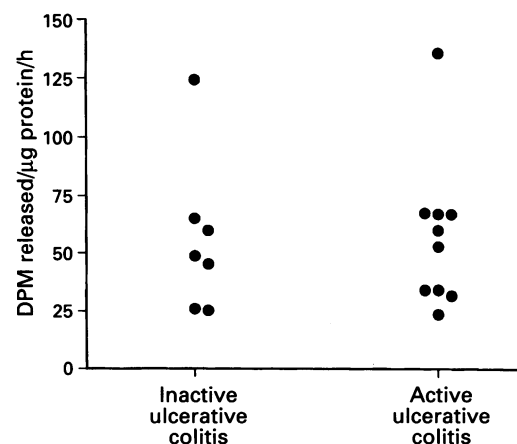


Figure 7: Comparison between faecal mucinase activity and disease activity in ulcerative colitis.

Potential treatments could therefore include bismuth subsalicylate, shown to have inhibiting activity in this study and for which promising therapeutic effects have been shown,<sup>14</sup> polyacrylates such as carbomer 934P, which have protease inhibiting activity,<sup>6</sup> and possibly the recently described sialidase inhibitors.<sup>23</sup>

ADD and HHT were supported by the National Association for Colitis and Crohn's disease and British Digestive Foundation. BJC is supported by the Medical Research Council.

- 1 Creeth JM. Constituents of mucin and their separation. *Br Med Bull* 1978; **34**: 17-24.
- 2 Miller RS, Hoskins LC. Mucin degradation in human colon ecosystems. Faecal population densities of mucin-degrading bacteria estimated by a 'most probable number' method. *Gastroenterology* 1981; **81**: 759-65.
- 3 Tsai HH, Sunderland D, Gibson GR, Hart CA, Rhodes JM. A novel mucin sulphatase from human faeces: its identification, purification and characterisation. *Clin Sci* 1992; **82**: 447-54.
- 4 Corfield AP, Williams AJK, Clamp JR, Wanger SA, Mountford RA. Degradation by bacterial enzymes of colonic mucus from normal subjects and patients with inflammatory bowel disease: the role of sialic acid metabolism and the detection of a novel esterase. *Clin Sci* 1988; **74**: 71-8.
- 5 Hoskins LC, Augustines M, McKee WB, Boulding ET, Kriaris M, Neidermeyer G. Mucin degradation in human colon ecosystems. Isolation and properties of faecal strains that degrade ABH blood group antigens and oligosaccharides from mucin glycoproteins. *J Clin Invest* 1985; **75**: 944-53.
- 6 Hutton DA, Pearson JR, Allen A, Foster SNE. Mucolysis of the colonic mucus barrier by faecal proteinases: inhibition by interacting polyacrylate. *Clin Sci* 1990; **78**: 265-71.
- 7 Samson HJ, Pearson JR, Allen A, Srivastava ED, Record CO. Significant increase in negatively charged serine dependent proteinases in ulcerative colitis. *gut* 1993; **34** (suppl 4): S10.
- 8 Rhodes JM, Black RR, Gallimore R, Savage A. Histochemical demonstration of desialation and desulphation of normal and inflammatory bowel disease rectal mucus by faecal extracts. *Gut* 1985; **26**: 1312-8.
- 9 MacDermott RP, Donaldson RM, Trier JS. Glycogen synthesis and secretion by mucosal biopsies of rabbit colon and human rectum. *J Clin Invest* 1974; **54**: 545-54.
- 10 Parker N, Finnie IA, Raouf AH, Ryder SD, Campbell BJ, Tsai HH, et al. High performance gel filtration using monodisperse highly cross-linked agarose as a one-step system for mucin purification. *Biomed Chromatogr* 1993; **7**: 68-74.
- 11 Harvey RF, Bradshaw JM. A simple index of Crohn's disease activity. *Lancet* 1980; **i**: 514.
- 12 Truelove SC, Witts LJ. Cortisone in ulcerative colitis. Final report on a therapeutic trial. *BMJ* 1955; **2**: 1041-8.
- 13 Lowry OH, Rosebrough NJ, Farr AL, Randall RJ. Protein determination with Folin-phenol reagent. *J Biol Chem* 1951; **193**: 265-75.
- 14 Ryder SD, Walker RJ, Jones H, Rhodes JM. Rectal bismuth subsalicylate as therapy for ulcerative colitis. *Aliment Pharmacol Ther* 1990; **4**: 333-8.
- 15 Ho SB, Neihans GA, Lyfogt C, Yan PS, Cherwitz DL, Gum ET, et al. Heterogeneity of mucin gene expression in normal and neoplastic tissue. *Cancer Res* 1993; **53**: 641-51.
- 16 Pullan RD, Thomas GAO, Rhodes M, Newcombe RG, Williams GT, Allen A, et al. Thickness of adherent mucus gel on colonic mucosa in humans and its relevance to colitis. *Gut* 1994; **35**: 353-9.
- 17 Collins JF, Thoman PJ, Shaw SL, Fine R. Studies on the elastolytic activity of chymotrypsin. *Connect Tissue Res* 1985; **13**: 291-8.
- 18 MacFarlane GT, Allison C, Gibson SAW, Cummings JH. Contribution of the microflora to proteolysis in the human large intestine. *J Appl Bacteriol* 1988; **64**: 37-46.
- 19 Van-Seuningen I, Davril M, Haymen A. Evidence for the tight binding of human mucus proteinase inhibitor to highly glycosylated macromolecules. *Biol Chem Hoppe Seyler* 1989; **370**: 749-55.
- 20 Mizon C, Yamani JE, Colombel J-F, Maes P, Balduyck M, Laine A, et al. Deglycosylation of  $\alpha_1$ -proteinase inhibitor is impaired in the faeces of patients with active inflammatory bowel disease (Crohn's disease). *Clin Sci* 1991; **80**: 517-23.
- 21 Burke DA, Axon AT. Hydrophilic adhesion of *Escherichia coli* in ulcerative colitis. *Gut* 1988; **29**: 41-3.
- 22 Bennet JD, Brinkman M. Treatment of ulcerative colitis by implantation of normal colonic flora [Letter]. *Lancet* 1989; **i**: 164.
- 23 von Itztein M, Wu W-Y, Kok GB, Pegg MS, Dayson JC, Jin B, et al. Rational design of potent sialidase-based inhibitors of influenza virus replication. *Nature* 1993; **363**: 418-23.